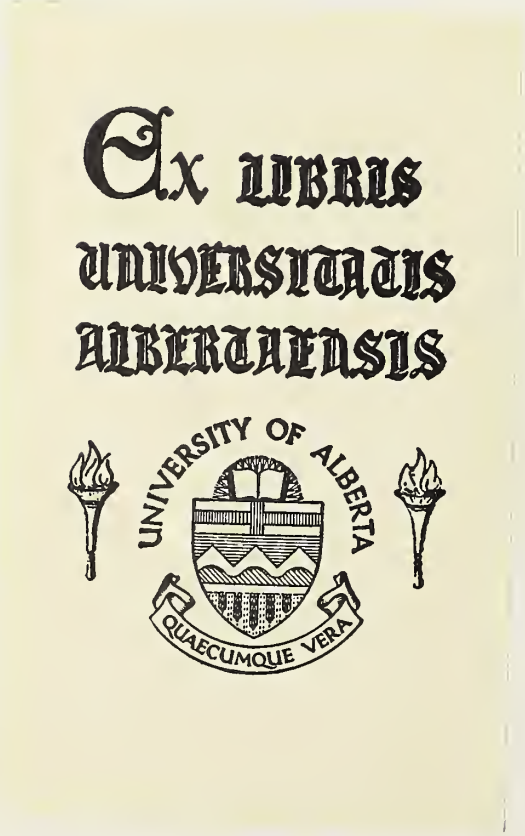

University of Alberta
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THE UNIVERSITY OF ALBERTA

GROWTH OF MYCELIUM AND PRODUCTION OF PYCNIDIA BY *PLENODOMUS MELILOTI* MARK.-LET.

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A THESIS

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ABSTRACT

The effects of light quality and intensity, carbohydrates, host materials, and soils on mycelial growth and pycnidium production of Plenodomus meliloti were studied in culture.

More abundant pycnidia were produced under continuous illumination of white, blue, and green than under gold light and in darkness. Generally ultraviolet light inhibited mycelial growth and pycnidium production of cultures. Pycnidia were also produced under very low light intensities of white and blue illumination.

A combination of glucose, fructose, and sucrose was found to be most favorable for pycnidium production. More pycnidia were produced when fructose was included singly or in combinations with other sugars. The fungus grew poorly and formed few pycnidia on water agar.

The autoclaved alfalfa root cortex was favorable for pycnidium production; whereas, the root stele was inhibitory for the pycnidium production. The fungus grew well on medium containing powdered roots of alfalfa, but produced few pycnidia. Fewer pycnidia were found on media containing plant extracts than on potato-sucrose agar medium though mycelial growth was comparable.

In shake culture, more mycelium was obtained in extracts of soils from Mile 1019, Yukon Territory than those from Lacombe,

Alberta. Also, more mycelium was obtained in extracts of soils collected in August than in May. Mycelial growth was greater in extracts of soils from the surface two-inch layer than from subsequent lower layers. In petri plate cultures, very small differences in mycelial growth and pycnidium production were observed between soils collected from Mile 1019 and Lacombe during May or August.

ACKNOWLEDGEMENTS

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INTRODUCTION

Plenodomus meliloti Mark.-Let. is a low-temperature fungus responsible for the disease known as brown root rot of legumes. It has been reported in the U.S.S.R. (38), Canada (13, 23, 31), Alaska (23), and Finland (39). This fungus is pathogenic on species of Medicago, Melilotus, and Trifolium (40), hollyhock (Althaea rosa) (37), red fescue (Festuca rubra), and Kentucky blue grass (Poa pratensis) (23), and found as a saprophyte on dead roots of Russian pigweed (Amaranthus retroflexus) and common oats (Avena sativa) (40).

The disease symptoms are characterized by brown, slightly sunken, necrotic lesions, which appear on roots of legumes. In these lesions are embedded numerous pycnidia. Sometimes only a few or even no pycnidia are observed (40). However, numerous pycnidia are also found on the lower parts of the stems and crowns of the native lupin (Lupinus arcticus) and occasionally on the lower stems of Medicago sativa and M. falcata (33).

Loss of legumes due to the disease occurs occasionally and is slight in central Alberta; whereas, destruction of legumes is frequent and very extensive in the Yukon Territory (33). Infection of host plants by P. meliloti under controlled laboratory conditions has not been too successful (31, 39, 40); however, Netolitzky (33) was successful in infecting legume and cereal seedlings grown in potato-sucrose agar. To date, the infection of inoculated greenhouse and field-grown plants has been accomplished only slightly (16, 33).

It appears that the fungus establishes itself during the fall, and disease symptoms become evident the following spring. Disease

symptoms and evidence of the fungus, in the form of pycnidia, are greatly reduced during the summer (31, 33).

One of the factors which may be responsible for the decrease of the fungus during the summer is the increase in temperature. In cultural studies, Sanford (40) found that the optimum temperature for growth of P. meliloti was 15° - 16°C with minimal growth occurring at 0° and 27°C. However, Netolitzky (33) found that cultures of four isolates, including that of Dearness and Sanford's (13), grew equally well at 5°, 15°, and 20°C. From Netolitzky's data it would appear that the optimum temperature for growth was much lower than 15°C.

The fungus has been cultured on a number of media (33, 39, 40). In this laboratory potato-sucrose agar was found to be most favorable for mycelial growth and pycnidium formation (33).

Since pycnidia are very seldom formed on the lower parts of stems, light may play an inhibitory role in pycnidium formation. Of course nutrition would be expected to affect formation of pycnidia. Therefore, these studies were initiated to determine the effects of light and some aspects of nutrition which included carbohydrates, host materials, and soils on the growth of mycelium and pycnidium formation.

ISOLATES

The isolates of P. meliloti used for these studies were similar to those used by Netolitzky (33). These included P_{10}, P_{15}, P_{20}, and P_{30}. Their origin and the hosts from which they were isolated are as follows:

Isolates	Hosts	Origin
P_{10}	Melilotus alba	Saskatoon, Saskatchewan
P_{15}	Melilotus sp.	Edmonton, Alberta
P_{20}	Medicago sp.	Dominion Experimental Farm, Mile 1019, Alaska Highway, Yukon Territory
P_{30}	Trifolium pratense	Finland

Isolates P_{10} was obtained from the Bureau of Schimmelcultures, Baarn, Netherlands, and P_{30} from Dr. A. Salonen, University of Helsinki, Finland. Isolates P_{15} and P_{20} were isolated by H. J. Netolitzky (33). Stock cultures of these isolates were maintained on slants of potato-sucrose agar (PSA) in the dark at 3°C.

Unless otherwise specified, inocula used throughout these studies were provided by discs (5mm diam.) taken from the outer margin of 10-day old colonies grown in petri plates on various media in the dark at 15°C.

EFFECTS OF LIGHT

Introduction

It is well known that light affects the morphology, pigmentation, phototropism, and reproduction of fungi (30). Coons (8) has reported that light is necessary for pycnidium formation of Plenodomus fuscomaculans, but it decreases mycelial growth in comparison with that in darkness. Leonian (24) found that P. destruens formed pycnidia both in darkness and under light.

Since the effects of light on P. meliloti are not known, studies were initiated to determine the effects of light quality and intensity on growth of mycelia and formation of pycnidia.

Materials and Methods

Preliminary experiments showed that there was no inhibition between colonies when isolates P_{10}, P_{15}, P_{20}, and P_{30} were grown on a single petri plate for 10 days. Therefore, discs of inocula (5mm diam.) were cut from colonies grown on PSA and placed in the center of each quarter of large petri plates (138mm diam.).

Cultures were grown at 15°C for 10 days under continuous white, blue, green, gold, or ultraviolet light. White, blue, green, and gold light, respectively, were supplied from G-E fluorescent lamps. Their absorption spectra* are shown in Fig. 1. Ultraviolet radiation (u.v.l.) was provided from a G-E germicidal ultraviolet lamp. The germicidal region of the u.v.l. was from 2000-3000 Å with the peak of the absorption spectrum being 2600 Å.

*From data compiled by the Lighting Institute, C.G.E. Co. Ltd., Toronto, Ontario, May 1955.

The light intensity was varied by placing the cultures at different distances from the light source. Measurements of light intensity (ft-c) for components of the visible spectrum, at the level of cultures, were made with a Weston Illumination Meter, Model 756. Cultures kept in the dark were considered as controls.

Four replicates were employed in each experiment. The diameter of each colony was measured after 10 days and rated with that of P_{30} grown in the dark. Degree of pycnidium production was rated on a scale of 0 - IV, with IV referring to numerous pycnidia and 0 to no pycnidia.

Results

Cultures in the dark for all isolates were cottony in nature with slight green pigmentation. The mycelial growth of P_{30} was greater than that of the other isolates.

Light quality--The results of mycelial growth and pycnidium production are shown in Fig. 2 and Table 1.

These isolates grew under each type of illumination; however, growth was significantly decreased under u.v.l. Isolate P_{10} was most affected under all forms of illumination resulting in a significant decrease in colony diameter.

Pycnidia were observed four days after inoculation under various forms of illumination and in darkness; however, none of these isolates produced pycnidia under u.v.l. Isolate P_{10} failed

to produce pycnidia under all conditions. Cultures of P_{15}, P_{20}, and P_{30} formed abundant pycnidia when exposed to white, blue, and green light as compared to the number produced on cultures kept in the dark. Pycnidium production under gold illumination was similar to that observed for cultures kept in the dark.

In addition to the effects on mycelial growth and pycnidium production, it was observed that pigmentation in the central area of all colonies was affected by light quality. Cultures incubated under white, blue, and green light were darker green in the middle than those under gold light and in the darkness. The centers of cultures, exposed to u.v.l., turned dark brown.

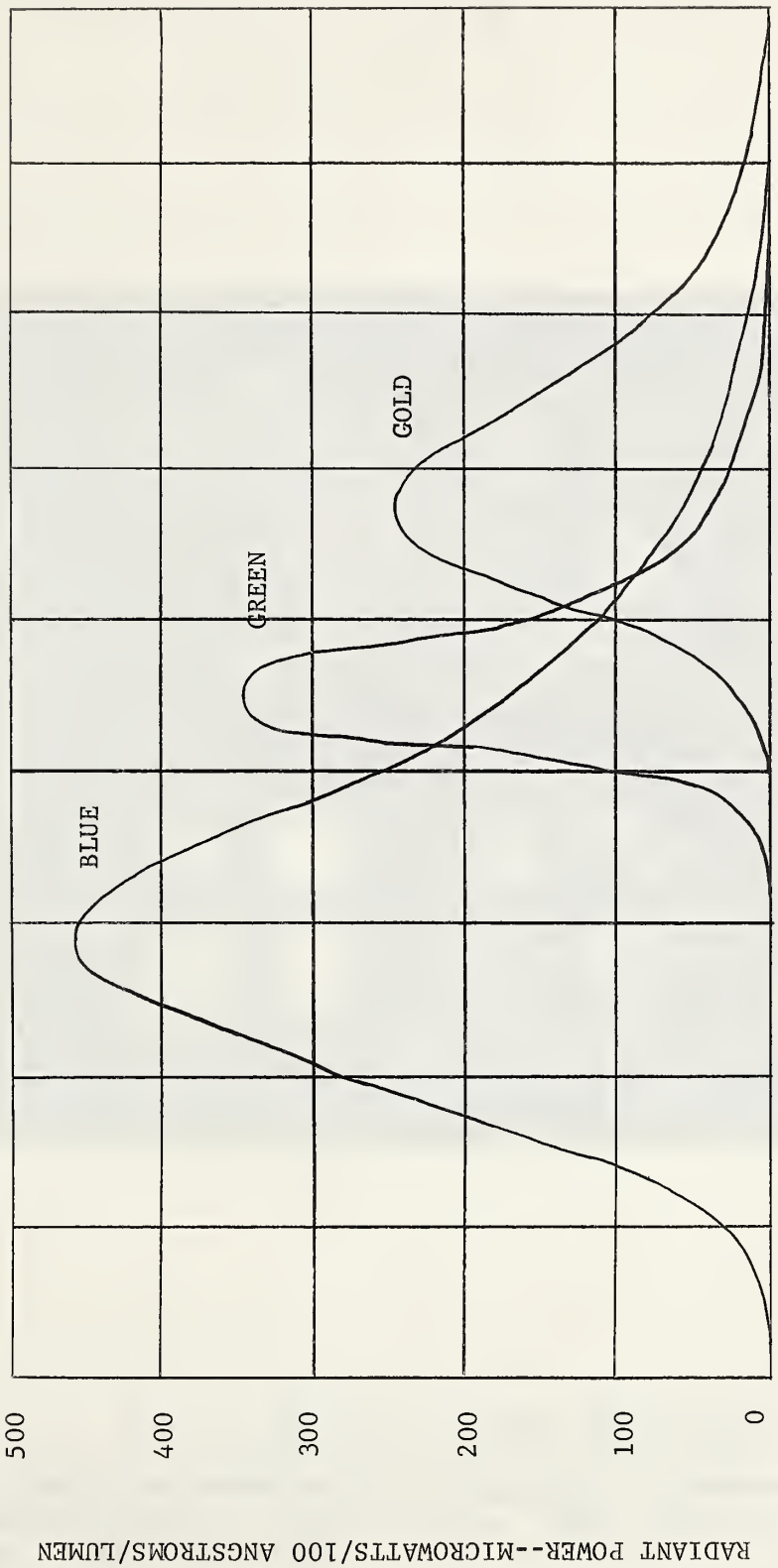

Fig. 1. Absorption spectra of blue, green, and gold light from G-E fluorescent lamps (From data compiled by the Lighting Institute, C.G.E. Company, Ltd., Toronto, Ontario, May 1955).

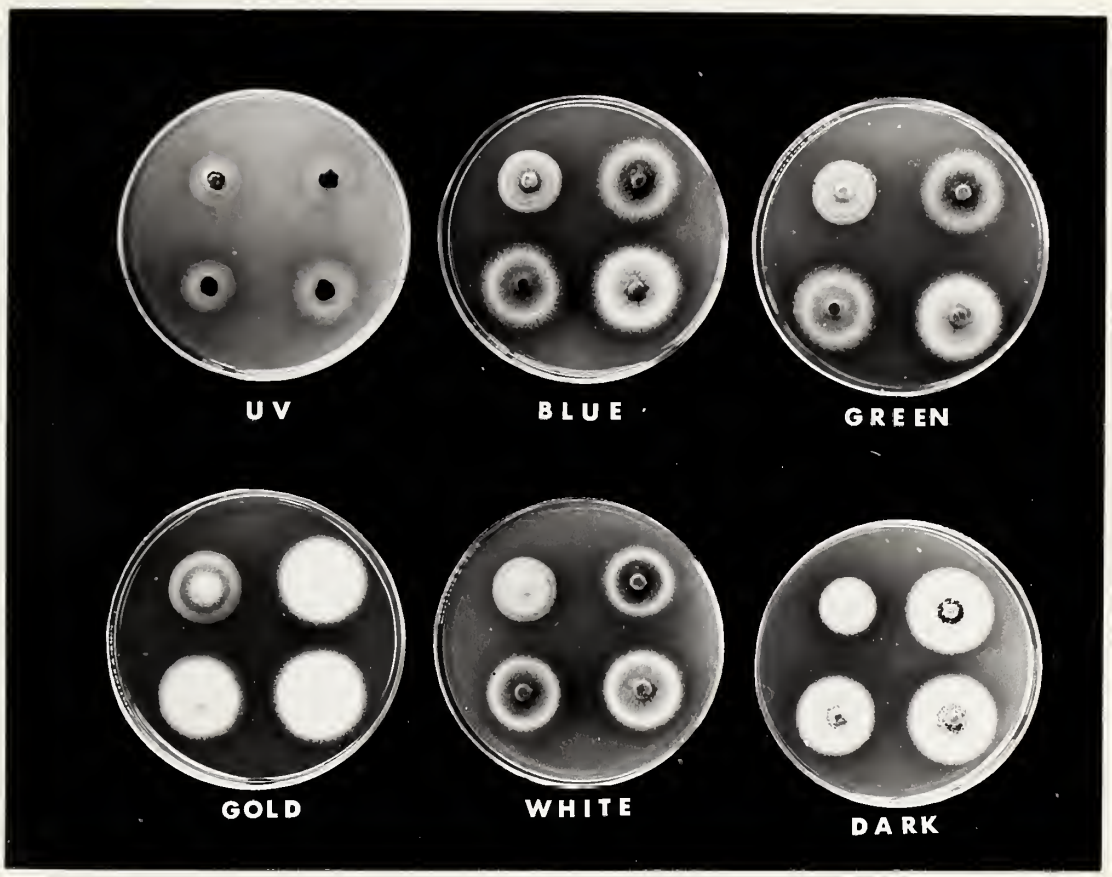

Fig. 2. Effects of darkness and continuous illumination of white, blue, green, gold, or ultraviolet light on mycelial growth and pycnidium production of P. meliloti. Arrangement of isolates in each petri plate: upper-left--P_{10}; upper-right--P_{15}; lower-left--P_{20}; lower-right--P_{30}.

Table 1. Effects of darkness and continuous illumination[a] of white, blue, green, gold, or ultraviolet light on mycelial growth and pycnidium production of P. meliloti on PSA at 15°C for 10 days.

Light	Relative growth[b]				Pycnidium production[c]			
	P_{10}	P_{15}	P_{20}	P_{30}	P_{10}	P_{15}	P_{20}	P_{30}
Ultraviolet	4.5[d]	5.1[d]	5.1[d]	6.2[d]	0	0	0	0
Blue	7.4	8.2	8.5	9.0	0	IV	IV	IV
Green	6.5[e]	9.2	9.3	9.5	0	III	IV	III
Gold	6.9[e]	9.7	9.4	9.8	0	I	II	II
White	6.9	8.3	8.7	9.1	0	IV	IV	IV
Dark (Control)	8.0	9.9	9.4	10.0	0	I	II	II

a Ultraviolet source 1.5 cm from the surface of cultures; 200 ft-c received from the other sources.
b Relative growth of isolates P_{10}, P_{15}, P_{20}, and P_{30} to P_{30} grown in darkness.
c Based on a scale of 0-IV in which 0 = no pycnidia and IV = numerous pycnidia.
d Significant difference at the 5% level within each isolate for various light qualities (LSD test).
e Significant difference at the 5% level within each light quality for various isolates (LSD test).

Light intensity--Studies on the effects of light quality showed that radiation from white and blue fluorescent lamps was stimulatory for pycnidium formation. Therefore, white, blue, and ultraviolet forms of light were selected to determine the effects of intensity on growth and pycnidium production. The results are presented in Fig. 3 and Table 2.

In Trial I and II, there was no significant difference in growth between isolates P_{15}, P_{20} or P_{30} under various light intensities. However, mycelial growth of P_{10} was significantly less than that of the other isolates. In Trial I, except for u.v.l., and in Trial II, there was no significant difference in growth within each isolate due to changes in light intensity.

Pycnidia were observed four days after inoculation under various intensities of white and blue light, but only after six days in cultures which were 7.5 cm from the u.v.l. source. Isolate P_{10} did not produce pycnidia at any light intensity. The other isolates did not produce pycnidia at distances of 1.5 and 5.0 cm from the u.v.l. source. Pycnidium production was not affected by changes in light intensities of white and blue illumination (Table 2--Trial I). Even at 10 ft-c of white light, there was a larger number of pycnidia as compared to cultures kept in the dark (Table 2--Trial II).

Green pigmentation, in the central part of all colonies, was more intense with increasing intensities of white and blue light. In cultures 7.5 cm from u.v.l. source, green pigmentation was observed in the central part of colonies. No such pigmentation was observed in cultures 1.5 and 5.0 cm from the u.v.l. source.

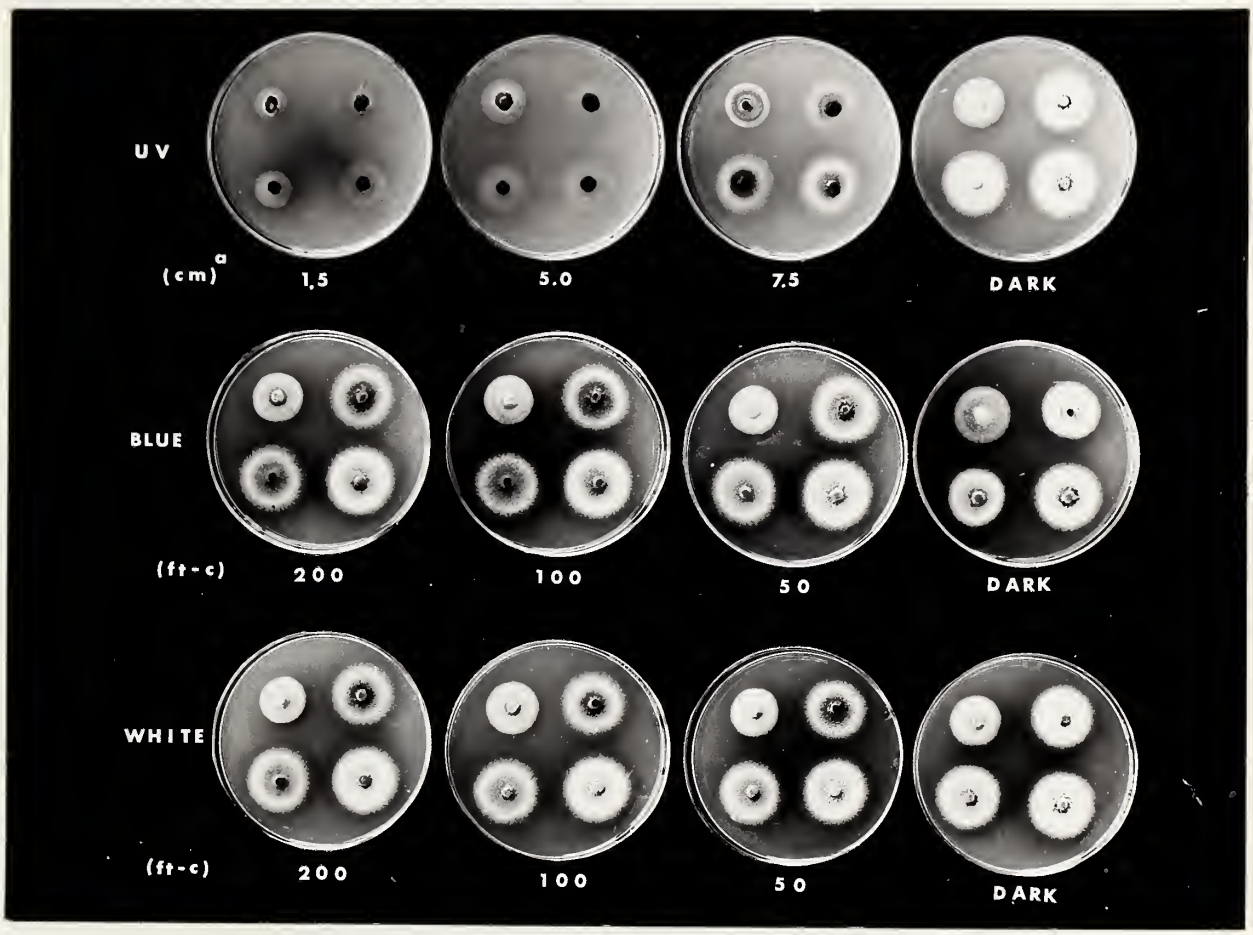

Fig. 3. Effects of darkness and light intensities of white, blue, or ultraviolet light on mycelial growth and pycnidium production of P. meliloti.

a Distance from the ultraviolet light source.

Arrangement of isolates in each petri plate: upper-left--P_{10}; upper-right--P_{15}; lower-left--P_{20}; lower-right--P_{30}.

Table 2. Effects of darkness and light intensities of white, blue, or ultraviolet light on mycelial growth and pycnidium production of P. meliloti on PSA at 15°C for 10 days.

Light	Relative growth[a]				Pycnidium production[b]			
	P_{10}	P_{15}	P_{20}	P_{30}	P_{10}	P_{15}	P_{20}	P_{30}
Trial I								
Ultraviolet								
1.5 cm[c]	4.7	3.4[d]	5.2[d]	5.4[d]	0	0	0	0
5.0 cm[c]	5.9	5.9[d]	7.3	7.4	0	0	0	0
7.5 cm[c]	6.1	8.3	8.7	9.3	0	III	III	III
dark	7.0	9.4	9.5	10.0	0	I	II	II
Blue								
200 ft-c	6.7[e]	9.4	9.4	9.8	0	IV	IV	IV
100 ft-c	6.6[e]	9.6	9.6	10.0	0	IV	IV	IV
50 ft-c	6.6[e]	10.0	9.7	10.0	0	III	IV	IV
dark	7.1[e]	9.3[e]	9.4	10.0	0	I	II	II
White								
200 ft-c	6.7[e]	9.6	9.5	9.8	0	IV	IV	IV
100 ft-c	7.3[e]	9.7	9.7	10.0	0	IV	IV	IV
50 ft-c	7.0[e]	9.6	9.6	10.0	0	IV	IV	IV
dark	7.5[e]	9.7	9.7	10.0	0	I	II	II
Trial II								
White								
50 ft-c	6.9[e]	9.4	9.1	9.8	0	IV	IV	IV
30 ft-c	6.6[e]	9.5	9.3	9.8	0	IV	IV	IV
10 ft-c	6.3[e]	9.3	9.4	9.8	0	III	IV	III
dark	5.9[e]	9.4	9.4	10.0	0	I	II	II

a Relative growth of isolates P_{10}, P_{15}, P_{20}, and P_{30} to P_{30} grown in the dark.
b Based on a scale 0-IV in which 0 = no pycnidia and IV = numerous pycnidia.
c Distance from the u.v.l. source.
d Significant difference at the 5% level within each isolate for various light intensities (LSD test).
e Significant difference at the 5% level within each light intensity for various isolates (LSD test).

Discussion

Light is absolutely required for pycnidium formation in some Sphaeropsidales (1, 8, 15). However, P. meliloti also initiated pycnidia in the dark. Pycnidia were produced more abundantly under white, blue, and green light than under gold light and in darkness. Similar results to these have also been reported for numerous fungi (2, 18, 43, 45, 47). Although some workers (17, 29, 42) have found that the blue region of the spectrum was effective for the induction of growth and reproductive structures, the results for P. meliloti indicate that the blue and green portions of the spectrum were most effective for pycnidium production.

Under natural conditions, light is not necessary for growth and pycnidium formation of P. meliloti since pycnidia are generally formed on roots some distance below the soil surface. The process(es) which leads to pycnidial production in culture receives some forms of stimulus from the lower part of the visible spectrum; whereas, this process(es) occurs naturally when the fungus is associated with roots in the dark. Coons (8) found that oxidizing agents, such as H_2O_2, could substitute partly for light. Such compounds were not used in these studies but should be considered.

Ultraviolet light is commonly used in culture work because of its germicidal properties. In these studies, high doses of u.v.l. repressed both mycelial growth and pycnidial production, but in low doses, it stimulated the production of pycnidia. Similar responses to u.v.l. radiation have been observed for other fungi (7, 22, 36, 42).

The significance of green pigmentation in growing cultures of P. meliloti is not known. However, it has been observed that the outer surfaces of immature pycnidia were light green and that the green pigmentation increased in intensity as the pycnidia matured. The nature of this green pigment and its role in association with light would be worth considering for further investigations.

EFFECTS OF CARBOHYDRATES

Introduction

Carbon compounds are used by fungi as the source of energy and as the chief structural element of organic compounds. Generally about one-half the dry weight of fungi is due to carbon (7, 27). Knowledge of carbon nutrition, therefore, is fundamental to an understanding of the physiology of fungi. Much valuable work has been done in this area for many fungi, but not for P. meliloti.

Since it is well known that the type of carbon source used strongly influences the growth of mycelium and the formation of the reproductive structures (10, 41, 44), several commonly used carbohydrates were selected to determine their effects on mycelial growth and pycnidium formation of P. meliloti.

Materials and Methods

Glucose, fructose, and sucrose used in these studies were the products of Difco Laboratories. They were sterilized using a Seitz filter and added to an approximately $55^{o}C$ solution of water agar (1.7%). Petri plates containing each medium were inoculated with mycelial discs taken from the colonies of P_{20} grown on water agar plates and incubated at $15^{o}C$ for 15 days.

Effects of glucose, fructose, and sucrose--Preliminary tests had shown that P. meliloti produced pycnidia more abundantly on autoclaved water-sucrose agar (2% sucrose) than on water agar. The auto-

claved sucrose solution was assumed to be partially hydrolyzed into glucose and fructose. These sugars were selected, therefore, to determine their relative suitability for growth and pycnidium formation. The above sugars, each at concentration of 8×10^{-2} M, were incorporated singly or in combinations with water agar (see above). Water agar, without added sugars, was used as control. Cultures were incubated in the dark at 15°C.

Effects of concentration--Since a combination of glucose, fructose, and sucrose resulted in the largest number of pycnidia (Table 3), the effects of different concentrations of this combination were determined. Media of water agar containing a combination of glucose, fructose, and sucrose at concentrations of 0, 2×10^{-2}, 4×10^{-2}, 6×10^{-2}, 8×10^{-2}, 10×10^{-2}, 12×10^{-2}, and 16×10^{-2} M respectively were prepared, inoculated with mycelial discs of P_{20}, and incubated in darkness at 15°C.

Effects of white light and carbohydrates--Previous experiments showed that white light (P. 9), and sugars (P. 17) increased pycnidium production. Therefore, a combination of white light and known sugars were employed. Continuous white illumination of 200 ft-c was supplied from G-E fluorescent lamps. Water agar containing a combination of glucose, fructose, and sucrose at a concentration of 8×10^{-2} M respectively was prepared and inoculated with P_{20} as above. Cultures kept at 15°C in darkness were used as controls.

Results

Effects of glucose, fructose, and sucrose--The effects of glucose, fructose, and sucrose on diameters of colonies and pycnidium production are shown in Table 3. Diameters of colonies on various sugars, singly or in combinations, were approximately the same, but higher than controls. However, the types of sugars and combinations of sugars had a marked effect on pycnidium production.

Fewer pycnidia were observed on the medium containing glucose than other treatments or control. The largest number of pycnidia per colony was observed on the medium containing a combination of glucose, fructose, and sucrose. On media containing fructose, more pycnidia were produced than for those containing glucose or sucrose respectively. On media containing a combination of two sugars, a higher number of pycnidia was obtained when fructose was included.

Table 3. Mycelial growth and pycnidium production of P_{20} on water agar containing various sugars.

Sugars (each sugar 8 x 10^{-2} M)	Diam. of colonies (mm)	Pycnidia/ colony
0 (Control)	61.4	1.8
Glucose	67.0	0.6
Fructose	66.5	12.8
Sucrose	66.0	4.4
Glucose + Fructose	66.4	17.2
Glucose + Sucrose	65.8	13.4
Fructose + Sucrose	65.2	18.4
Glucose + Fructose + Sucrose	64.6	54.6

Each result is the average of 5 replicates repeated twice.

Effects of concentrations--The effects of concentrations of glucose, fructose, and sucrose used in combination are shown in Fig. 4. The concentrations of sugars did not affect the linear growth of colonies noticeably; however, pycnidium production was greatly affected. The greatest number of pycnidia was observed when the concentration was 8×10^{-2} M, and decreased at higher and lower concentrations.

Effects of white light and carbohydrates--The effects of white-light illumination and sugars on mycelial growth and pycnidium production are shown in Table 4. Diameters of mycelial colonies under continuous white light and in darkness were similar; however, white light stimulated significantly the production of pycnidia on water agar and medium containing glucose, fructose, and sucrose. The increase in pycnidium production was approximately 25 times greater on water agar than on the medium containing the combination of sugars. It should be noted here that on PSA, under white light, the numbers of pycnidia per colony were too numerous for counting (P. 9).

Green pigmentation was observed in the central portion of the colonies under white light illumination. Colonies grown on the medium containing the sugars had a larger pigmented area than those grown on water agar.

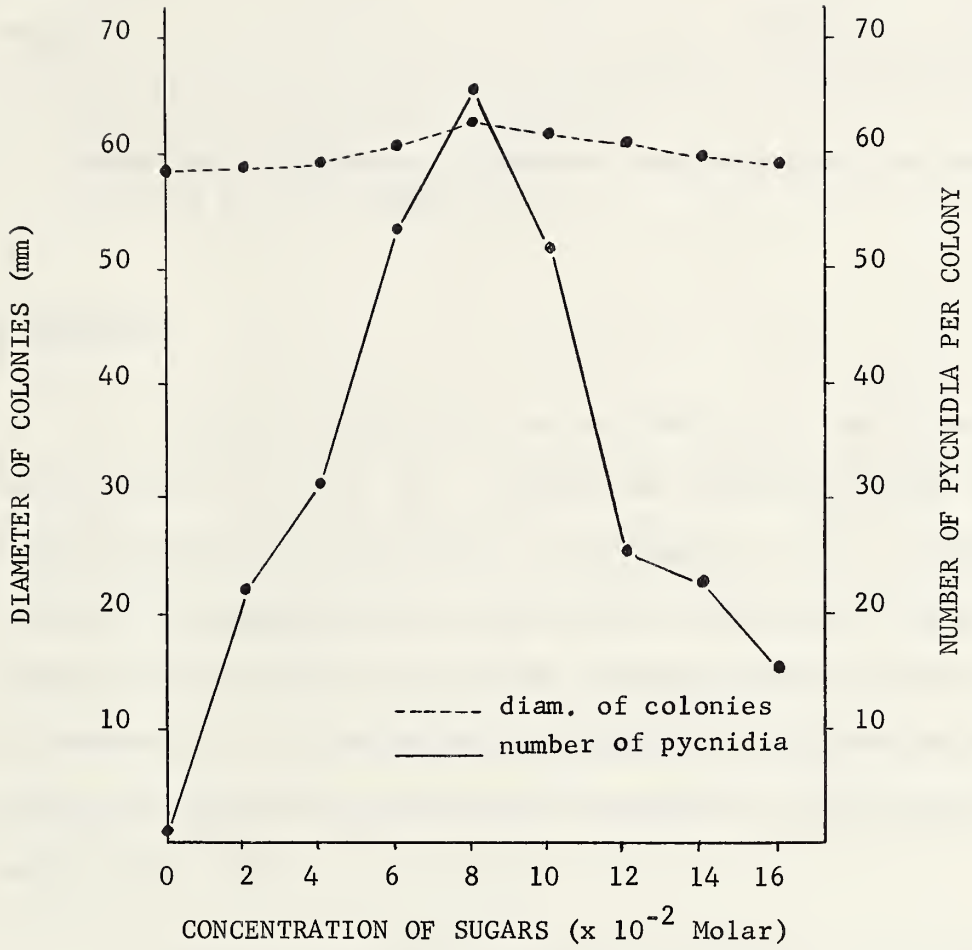

Fig. 4. Effects of concentrations of sugars[a] on mycelial growth and pycnidium production of P_{20}.

a A combination of glucose, fructose, and sucrose at the concentration indicated.

Table 4. Effects of white light illumination[a] and sugars on mycelial growth and pycnidium production of P_{20}

Media	Diam. of colonies (mm)		Pycnidia/ colony	
	White light	Dark	White light	Dark
Water agar	59.4	61.3	258.4	1.2
Sugars[b]	61.0	63.7	523.0	54.4

a Continuous illumination at 200 ft-c.
b A combination of glucose, fructose, and sucrose at the concentration of 8×10^{-2} M respectively.

Discussion

Glucose is reported to be the best carbon source for growth in most fungi (6, 7, 27, 44). Glucose and sucrose have been shown to be favorable for sporulation in some fungi (6, 27). In these studies, P. meliloti showed no appreciable differences in its mycelial growth on medium containing glucose, fructose, and/or sucrose singly or in combinations. On equimolar concentrations of glucose and sucrose, Sordaria fimicola increased growth in comparison with either of these sugars alone (26).

Although very little variation in mycelial growth was obtained in various treatments on petri plate cultures, it is possible that differences in the amount of mycelium, using shake-culture technique, would be obtained.

It is evident that P. meliloti is able to grow and produce pycnidia on extremely small amounts of available carbohydrates since, under white light, a large number of pycnidia was observed on a medium

composed of water agar. Coons (8) found that P. fuscomaculans was able to grow and produce pycnidia on filter paper in water under diffuse daylight.

Agar contains D-galactose and 3, 6-anhydro-L-galactose (19) which may be available to the growing fungus. On the other hand, P. meliloti may secrete an enzyme which hydrolyzes the sugar sufficiently for growth and pycnidium production. The effects of galactose on mycelial growth and pycnidium production were not determined.

The role of single or combinations of sugars in pycnidium production cannot be explained at this time. It is strange that glucose, commonly used in culture medium, inhibited the production of pycnidia, but glucose is commonly found in plant extracts.

As mentioned previously in these studies (P. 9), white light plays an important role in increasing production of pycnidia. This fact was emphasized in these experiments using a combination of light and sugars. The results of these experiments indicate that much fundamental work is necessary to explain the function of light in association with nutritional requirements and role of nutrients in pycnidium production.

EFFECTS OF HOST MATERIALS

Introduction

Netolitzky (33) found that PSA was the best of various media used for mycelium growth and pycnidum formation of P. meliloti. He also found that mycelial growth and pycnidium formation were less on agar media containing water extracts from roots, stems, and leaves of Medicago and Melilotus species, but on autoclaved intact roots numerous pycnidia were formed. El-Tobsby and Sinclair (14) reported that water extracts from epicarp and mesocarp tissues of orange inhibited the growth of Phomopsis citri significantly greater than an extract from the endocarp. Inhibition of germination of spores of Fomes igniarius by extracts from host tissue has also been reported by Wall (46).

It is expected that host plant(s) would play an important role in the growth and pycnidium formation of P. meliloti. Therefore, these investigations were made to determine the effects of root sections, root powder, and extracts from host materials on mycelial growth and pycnidium formation.

Materials and Methods

Roots and root powder--Alfalfa roots were collected near Ellerslie, Alberta in June and August, 1965. The variety of the field-grown Medicago sp. is unknown. The roots (approx. 9 mm diam.) were washed with tap water, rinsed with distilled water, and cut into 1-cm lengths. The sections were split in half longitudinally; some

were separated into 2 parts consisting of stele and cortex and the cortex was cut into 1-cm squares. These pieces were sterilized by autoclaving at 121.6°C for 1 hour and embedded in petri plates containing water agar.

The alfalfa root powder was prepared by oven-drying clean roots at 95°C for 48 hours and ground with a Intermediate Mill so that the root powder passed through a 60-mesh screen. For preparing root powder media, water agar (1.7%) was used as the basal medium in which the root powder was supplied in amounts of 0, 0.1, 0.5, 1.0, 1.5, and 2.0% respectively. Media were sterilized at 121.6°C and 15 lb pressure for 20 minutes.

Inocula consisted of mycelial discs (5mm diam.) of P_{20} taken from 10-day old colonies grown on agar media containing 0.1% root powder. Five replicates were used in each experiment. Cultures were kept in the dark at 15°C for 15 days.

Root and leaf extracts--Roots and leaves of sweet clover, variety Erector, were obtained at the Dominion Experimental Farm, Lacombe, Alberta in July, 1964. The roots and leaves were washed with tap water and rinsed with distilled water. After removing the stele, the cortex was cut into 1 cm lengths. Approximately 500 g of the cortical pieces were immersed in 2.5 liters of 95% ethyl alcohol at room temperature for 20 hours. The ethanol extract was filtered through Eaton-Dikeman No. 613 filter paper and the filtrate combined with subsequent filtrates. The cortical tissue was ground in a Waring Blendor after addition of approximately 2 liters of 80% ethanol. The homogenate was let stand for 20 hours and again filtered through

Eaton-Dikeman filter paper. This filtrate was combined with the first ethanol filtrate. The residue was discarded.

The combined filtrate was concentrated to approximately 225 ml under vacuum at 52° - 54°C with a Flash-Evaporator. The extract was cleared by centrifuging at 5400 xg for 10 minutes using a Servall Superspeed Centrifuge. The cleared supernatant was decanted and made up to 225 ml with deionized water. 25 ml of isopropanol was added to prevent the growth of microorganisms and esterification of the amino acids.

The extract was divided into equal parts. One half will be referred to as 'complete', i.e., no analysis or separating procedure was employed. With the other half, the amino acids were removed using Dowex 50W - x 8 ion-exchange resin following the procedure of Plaisted (35). In order that subsequent media would have the same concentration of nitrogen, the total nitrogen contents of the 'complete' extract and the amino-acid fractions were determined using the micro Kjeldahl procedure (25). The 'complete' extract and the amino-acid fractions were evaporated to near dryness at 52° - 54°C using the Flash-Evaporator. Deionized water was added to each so that the final concentration of nitrogen was 0.025%. This concentration was used because the total nitrogen of potato extracts, generally used for culture medium, was found to be 0.029 per cent. Two per cent sucrose was added to the amino-acid fractions. Both the 'complete' extract and the amino-acid fractions were combined with agar (1.7%) respectively, sterilized by autoclaving at 121.6°C for 20 minutes, and poured into 138 mm diam. petri plates.

Inoculation procedure was similar to that described on P. 4. Cultures were incubated at 15°C in darkness and under continuous white light illumination of 70 ft-c for 10 days. Cultures grown on PSA were used for comparison.

To analyze for free amino acids in the amino-acid extract, two-dimentional chromotography using Whatman No. 1 filter papers was employed. Butanol-acetic acid-water (4:1:5) was the first irrigating solvent and phenol-water (4:1) the second. The amino acids were detected after the chromatograms were sprayed with a solution of 1% ninhydrin in 95% ethyl alcohol (3). To analyze for sugars, a one-dimensional paper chromatographic technique was used with butanol-acetic acid-water being the irrigating solvent. Aniline hydrogen phthalate (3), sprayed on dried chromatograms, was used for the detection of sugars.

The above procedures were repeated using leaves of sweet clover.

Results

Root sections--The results of mycelial growth and pycnidium production on autoclaved roots are presented in Fig. 5 and Table 5.

Mycelial growth was only slightly less on the pieces of stele; however, pycnidium production was markedly lower compared to growth and pycnidium production on the root and cortical pieces. A larger number of pycnidia occurred on the cortex alone, in comparison with those on the whole root. A few small pycnidia were produced on the water agar adjacent to the root pieces.

Table 5. Effects of autoclaved alfalfa root pieces embedded in water agar on mycelial growth and pycnidium production by P_{20}.

Substrate	Radius of colony (mm)	Pycnidia/ root piece
Whole root	27.7	62.2
Cortex	26.4	80.2*
Stele	26.0	4.8**

* Significant difference at the 5% level (LSD test).
** Significant difference at the 1% level (LSD test).

Root powder--On the alfalfa root powder-agar, the mycelial growth of P_{20} varied with the seasons of which the roots were collected (Fig. 6). Diameter of mycelial colonies was larger on media containing root powder collected in June than on material collected in August. The diameter of colonies decreased with increasing concentrations; however, the aerial growth of mycelium appeared to increase with increasing root powder concentrations.

Differences in pycnidium production due to root powder concentration or to time of root sampling were not apparent.

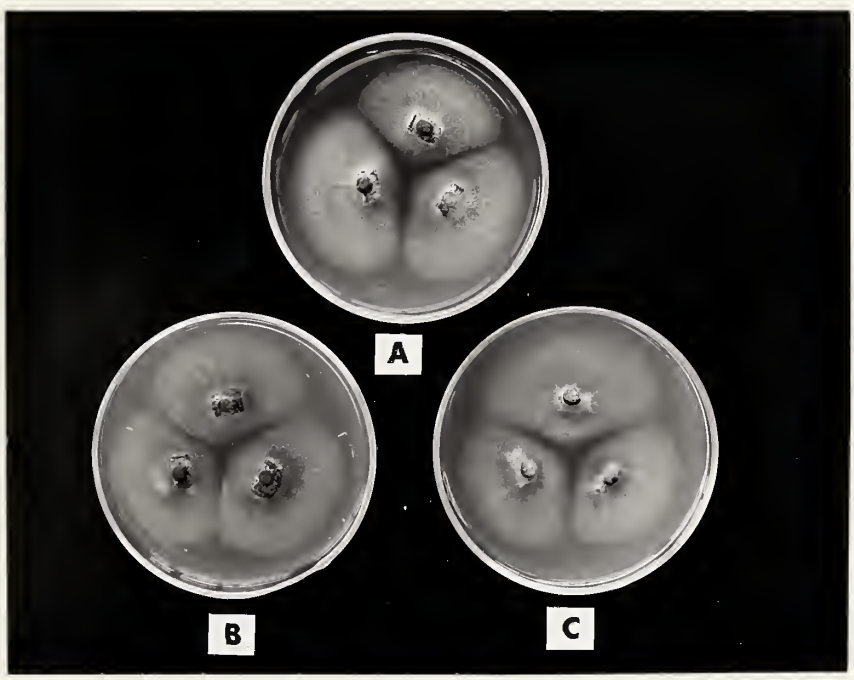

Fig. 5. Growth and pycnidium production of P_{20} on autoclaved sections of alfalfa root embedded in water agar. A. whole root; B. cortex; C. stele.

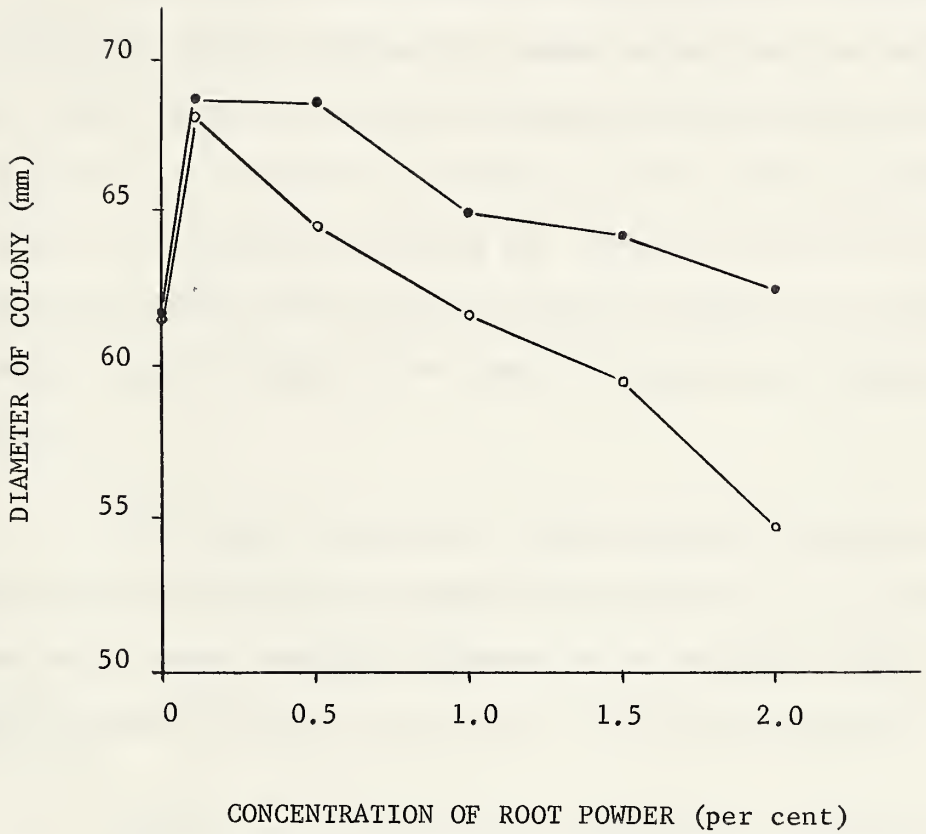

Fig. 6. Effects of various concentrations of alfalfa root powder on mycelial growth of P_{20}. Roots collected in June (—— • ——) and August (—— o ——).

Root and leaf extracts--The effects of root and leaf extracts are shown in Table 6. Mycelial growth on root and leaf extracts was less than that observed on PSA. All isolates grew faster on medium containing amino-acid extracts with added sucrose than on medium containing only the 'complete' extract.

In the dark, fewer pycnidia were produced on medium containing amino-acid extract with added sucrose and leaf extracts. Very small differences could be observed between numbers of pycnidia produced on the 'complete' root extract and PSA. More pycnidia were produced on the medium containing the 'complete' extract of roots than on the medium containing the amino-acid extract plus sucrose; however, mycelial growth on the former was poorer than that on the latter.

White light illumination reduced mycelial growth on PSA, but did not affect the growth on the various plant extracts. Under white light, pycnidia produced on PSA were much higher than those on medium containing any of the root and leaf extracts.

Isolate P_{10} did not produce pycnidia as a result of any treatment. Isolates P_{20} and P_{30} did not form pycnidia on medium containing the amino-acid extract plus sucrose in the dark or in the light.

Amino compounds and sugars detected in the ethanol extracts of sweet clover roots and leaves shown in Table 7 included aspartic acid, glutamic acid, alanine, lysine, leucine/isoleucine, valine,

proline, tyrosine, threonine, gamma-aminobutyric acid, serine, glycine, phenylalanine, glutathione, glucose, and sucrose. Two and three unknown ninhydrin-reacting spots were found on chromatograms of leaf and root extracts respectively. Asparagine and glutamine were not detected in leaf extracts.

Glucose and sucrose were detected in root extracts; however, only glucose was detected in leaf extracts.

Table 6. Effects of sweet clover root and leaf extracts on mycelial growth and pycnidium production of Plenodomus isolates under white light and in darkness.

Media	Relative growth[b]				Pycnidium production[c]			
	P_{10}	P_{15}	P_{20}	P_{30}	P_{10}	P_{15}	P_{20}	P_{30}
Dark								
'Complete' root extract	4.6	3.7	4.2	4.2	0	II	I	II
Roots--amino-acid fractions plus sucrose	6.1	5.5	6.2	6.6	0	I	0	0
'Complete' leaf extract	4.9	6.2	5.6	5.7	0	I	I	I
Leaves--amino-acid fractions plus sucrose	6.3	7.3	7.6	8.2	0	I	I	I
PSA	6.3	9.7	9.3	10.0	0	II	II	II
Light[a]								
'Complete' root extract	4.0	4.1	4.6	4.2	0	I	0	II
Roots--amino-acid fraction plus sucrose	5.2	6.2	6.4	7.2	0	I	0	0
'Complete' leaf extract	5.0	6.2	6.2	6.2	0	I	I	I
Leaves--amino-acid fraction plus sucrose	5.7	7.3	6.6	8.0	0	I	I	I
PSA	5.9	7.5	7.8	8.4	0	III	III	III

a Continuous illumination of white light--70 ft-c.

b Relative growth of isolates P_{10}, P_{15}, P_{20}, and P_{30} to P_{30} grown in darkness.

c Based on a scale of 0-IV in which 0 = no pycnidia and IV = numerous pycnidia.

Table 6. Effects of sweet clover root and leaf extracts on mycelial growth and pycnidium production of Plenodomus isolates under white light and in darkness.

Media	Relative growth[b]				Pycnidium production[c]			
	P_{10}	P_{15}	P_{20}	P_{30}	P_{10}	P_{15}	P_{20}	P_{30}
Dark								
'Complete' root extract	4.6	3.7	4.2	4.2	0	II	I	II
Roots--amino-acid fractions plus sucrose	6.1	5.5	6.2	6.6	0	I	0	0
'Complete' leaf extract	4.9	6.2	5.6	5.7	0	I	I	I
Leaves--amino-acid fractions plus sucrose	6.3	7.3	7.6	8.2	0	I	I	I
PSA	6.3	9.7	9.3	10.0	0	II	II	II
Light[a]								
'Complete' root extract	4.0	4.1	4.6	4.2	0	I	0	II
Roots--amino-acid fraction plus sucrose	5.2	6.2	6.4	7.2	0	I	0	0
'Complete' leaf extract	5.0	6.2	6.2	6.2	0	I	I	I
Leaves--amino-acid fraction plus sucrose	5.7	7.3	6.6	8.0	0	I	I	I
PSA	5.9	7.5	7.8	8.4	0	III	III	III

a Continuous illumination of white light--70 ft-c.

b Relative growth of isolates P_{10}, P_{15}, P_{20}, and P_{30} to P_{30} grown in darkness.

c Based on a scale of 0-IV in which 0 = no pycnidia and IV = numerous pycnidia.

Table 7. Amino compounds and sugars of ethanol extracts of sweet clover roots and leaves detected by paper chromatographic methods.

Compounds	Roots	Leaves
Amino compounds		
Aspartic acid	+	+
Glutamic acid	+	+
Glutamine	+	-
Alanine	+	+
Lysine	+	+
Leucine/isoleucine	+	+
Valine	+	+
Proline	+	+
Tyrosine	+	+
Threonine	+	+
Gamma-aminobutyric acid	+	+
Serine	+	+
Asparagine	+	-
Glycine	+	+
Phenylalanine	+	+
Glutathione	+	+
Unknown A	+	-
Unknown B	+	-
Unknown C	+	-
Unknown D	-	+
Unknown E	-	+
Sugars		
Glucose	+	+
Sucrose	+	-

+, presence; -, not detected.

Discussion

Autocalved stele of alfalfa roots significantly inhibited pycnidium production of P. meliloti; whereas, pycnidia were produced abundantly on autoclaved cortical pieces. It would appear that some natural constituents in the stele play an important role in preventing pycnidium production or that the stele does not contain those factors which could support or promote pycnidium production. The stele does contain sufficient nutrients to support mycelial growth since the linear growth of mycelium in the petri plates containing stele and cortical pieces respectively was nearly the same. The effects of non-autoclaved pieces of stele would be of much interest for further studies.

Colonies of larger diameters were observed on medium containing powdered roots of alfalfa collected in June than in August. These results coincide with the field observation made by Netolitzky (33) and McDonald (31). They found that the occurrence of P. meliloti in the Yukon Territory and in Manitoba, respectively, was extensive and severe, but greatly reduced during the latter part of the summer.

It was observed that the natural-occurring sugars in host roots are important factors influencing pycnidium production. In these studies (P.17), a combination of glucose, fructose, and sucrose has been found to be the most favorable source of carbohydrate for the pycnidium production of P. meliloti. Fructose was not detected in the host roots collected in summer. A similar result was obtained by Wilding et al. (49); however, they detected fructose, glucose, and

sucrose in alfalfa roots sampled in December. Therefore, it would be expected that more pycnidia would be produced on extracts of host roots collected in winter.

In these studies it was generally found that medium containing agar, agar containing sucrose, agar containing various root extracts, respectively, produced fewer pycnidia than on PSA or on autoclaved cortical pieces of alfalfa roots. It would appear, therefore, that other factors than carbon play an important role in pycnidium production. These factors may include a specific nitrogen compound or combinations of compounds, vitamins, etc.

EFFECTS OF SOIL EXTRACTS

Introduction

Soil contains amino acids, fats, organic acids, carbohydrates, other organic as well as inorganic compounds (20). Of these compounds, more than 30 amino-acids and several carbohydrates have been isolated from acid hydrolysates of soils using paper chromatographic techniques (11, 21). Although none or only trace amounts of amino acids have been detected in water or ethanol extracts of soils (9, 34), such soil extracts have been found to be a good culture medium for the study of some microorganisms (28, 32).

Under natural conditions, P. meliloti grows and forms pycnidia, generally on the roots of legumes. It is expected that soils, in some way, could affect mycelial growth and pycnidium formation of the fungus. Therefore, these studies were initiated to determine whether water extracts from soils of different depths, locations, and seasons at which the soils were sampled would have any effect on mycelial growth and pycnidium formation.

Materials and Methods

Soil samples were taken from the plots of sweet clover at the Dominion Experimental Farms located at Lacombe, Alberta, and at Mile 1019, Alaska Highway, Yukon Territory, in May* and July 1965 respectively. Six soil samples, each of which successively represented

*The May samples from Mile 1019 were obtained in 1963 and stored in plastic bags at -23.3°C.

a 2-inch layer from the soil surface, were used in these studies. The moist soils were air-dried for 2 days and passed through a 1 mm sieve to get a homogeneous soil sample and to remove large pieces of debris. Weights of the air-dried soil samples, used for extraction, were corrected on an oven-dry weight basis.

Following the Lochhead and Chase procedure (28), soil extracts were prepared by autoclaving 1 kg soil (oven-dry weight basis) with 1 liter distilled water at 121.6°C for 30 minutes, filtered through Eaton-Dikeman No. 617 and Whatman No. 2 filter papers respectively, and final volumes made up to 2 liters with distilled water. One half of this extract was incorporated with 1.7% agar and the other half was used as a medium for shake culture. No sugars were added.

Inoculum for petri plate cultures consisted of P_{20} grown on the soil extract agar. For shake cultures, a 1-ml pycnospore suspension of P_{20} (30-50 pycnospore/ field at 400 x magnification) was placed in sterilized 250-ml Erlenmeyer flasks containing 40 ml of aqueous soil extract. Petri plates and shake cultures were incubated in darkness at 15°C for 15 days. Five replicates were used in each experiment. Petri plates containing PSA and shake cultures with potato-sucrose broth were included for comparison.

Diameters of colonies on petri plates were measured and measurements are shown in Fig. 7. Mycelial mats from shake flasks were filtered through previously tared Eaton-Dikeman No. 615 filter

paper and washed extensively with distilled water to remove traces of medium. Mycelial mats were dried at 95°C for 24 hours, and the dry weight of mycelium obtained. Dry weights of mycelium are shown in Fig. 7.

Using spot tests, approximately 200 ul of the extracts from the surface and bottom soil samples only were analyzed for amino acids and sugars. One per cent ninhydrin in 95% ethyl alcohol and aniline hydrogen phthalate were used to detect amino acids and sugars respectively (3).

Results

Trace amounts of amino acids and no sugars were detected using ninhydrin and aniline hydrogen phthalate respectively. No efforts were made to determine which amino acids were present in the soil extracts.

An average of 364 mg dry-weight of mycelium per shake flask was obtained when P_{20} was grown in potato-sucrose broth for 15 days. The average diameter of colonies of P_{20} was 64.2 mm grown in petri plates on PSA for 15 days.

The amounts of mycelial growth obtained using soil extracts in shake flasks were extremely low as compared with the amounts obtained using potato-sucrose broth (Fig. 7). Generally P_{20} produced more mycelium in flasks containing extracts of soils from Mile 1019, Yukon Territory than from Lacombe, Alberta. Also, there was a decrease in mycelial growth in extracts from the surface layer to the lowest

two-inch layer. More mycelial growth was obtained in extracts of soils collected in August than in May.

In petri plate cultures, very small differences in growth were observed between soils collected from Mile 1019 and Lacombe during May or August (Fig. 7). Diameters of colonies grown on soil extracts of Lacombe and Mile 1019 were very similar to those obtained on PSA. However, the type of mycelial growth and number of pycnidia on PSA were significantly different. On soil extracts colonies were thin, grayish white with only a few small pycnidia being formed; whereas, on PSA colonies were very dense, grayish green and aerial with numerous large pycnidia.

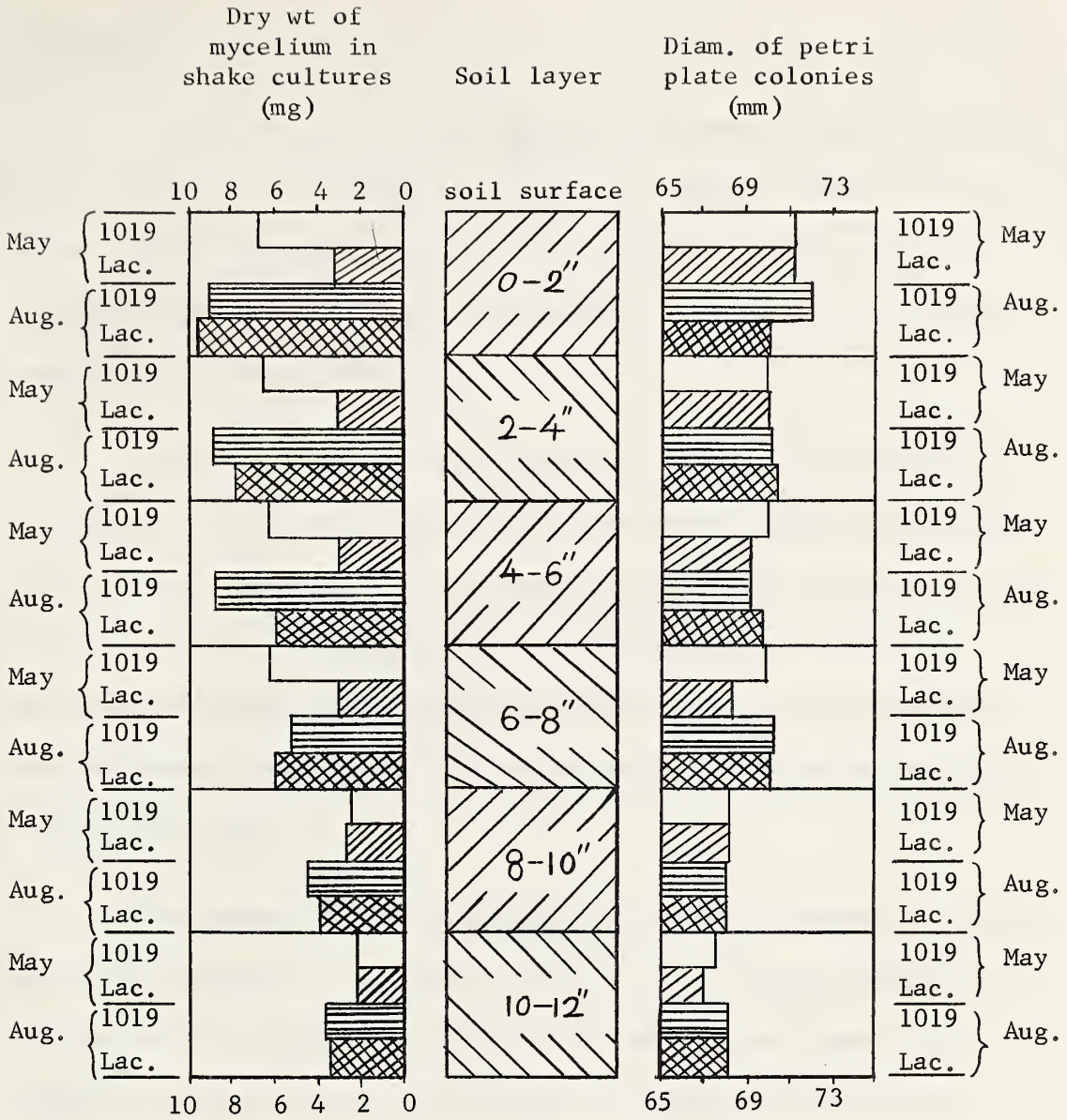

Fig. 7. Effects of aqueous extracts from 2" layers of soil sampled at Dominion Experimental Farms, Mile 1019, Alaska Highway, Yukon Territory and Lacombe, Alberta, respectively, during May and August on mycelial growth of P_{20} in shake and petri plate cultures.

1019--Mile 1019, Alaska Highway, Yukon Territory.
Lac.--Lacombe, Alberta.

Discussion

The petri-plate culture technique cannot be used to measure the availability of nutrients to a fungus as can the shake-culture method. This is borne out in Fig. 7, which shows little variation in growth of mycelium in petri plate cultures as compared to growth of mycelium in shake flasks.

Although the spot tests indicated that the concentration of sugars and amino acids were very low, mycelial growth was obtained in petri plate cultures. It is possible that if the soil extracts used for shake-culture studies had been concentrated more growth would have been obtained. Since sugars were not detected, it is presumed that the fungus was able to utilize the carbon from the amino acids or agar for growth.

The prevalence of P. meliloti is generally slight in Alberta and often extensive in the Yukon (33), but it is greatly reduced in Manitoba and the Yukon during the latter part of the summer (31, 33). However, in these studies, it was found that mycelial growth on extracts from soils sampled in August was greater than from soils sampled in May. This may be due to the fact that more available nutrients in the form of root exudates would be extracted in August.

Soil microbes populate much more in the upper layers of the soil than in the lower layers (5). These soil fungi are supported as the result of decomposition of organic matter (48). It is to be expected that the microorganism population would be higher in that part of the soil which contains the highest organic matter.

The upper layers of the Yukon soil used in these studies are higher in organic matter than the lower layers (12). The top twelve inches of soil from Lacombe is uniform (4) and higher in organic matter than the soil from Mile 1019. The amounts of organic matter, however, would not seem to play an important role in the greater prevalence of P. meliloti in the Yukon than in the Lacombe regions, since growth of mycelium, in shake flasks, was nearly always greater in those extracts of soils from the Yukon than from Lacombe. It would appear that some factor(s) in the Lacombe soil is inhibitory for the growth of this fungus or that a factor(s) in the soils of the Yukon Territory is favorable for growth. Under field conditions, one of the factors which could prevent the growth of P. meliloti in the Lacombe soil may be the direct effects of competitive microorganisms.

Although not expected, extracts from various depths of the Lacombe soils obtained in August had a noticeable effect on the growth of mycelium. This is surprising since the Lacombe soil was of one type to a twelve-inch depth; on the other hand, the soil of Mile 1019 shows a gradation in texture and structure from the surface to the lowest layer.

Further studies on the effects of soil extracts on growth of P. meliloti need to be carried out.

SUMMARY

1. Pycnidium production occurred more abundantly under continuous white, blue, and green than under gold light and in darkness. No significant differences in mycelial growth were observed under various light qualities and in darkness.

2. The fungus grew well on medium containing various sugars singly or in combinations. A combination of glucose, fructose, and sucrose was most favorable for pycnidium production. Of these sugars, fructose was best for pycnidium production.

3. The autoclaved alfalfa root cortex was favorable and the root stele inhibitory for the pycnidium production.

4. The fungus grew well on medium containing powdered roots of alfalfa, but produced few pycnidia.

5. Naturally occurring sugars in host roots were found to be important in increasing the pycnidium production.

6. Mycelial growth on the medium containing leaf extracts of sweet clover was superior to those of root extracts.

7. Mycelial growth was less in the extracts of soils from Lacombe, Alberta than Mile 1019, Alaska Highway, Yukon Territory. Also, less growth was observed in the extracts of soils collected in May than in August. More mycelium was obtained in the extracts of soils from the surface two-inch layer than subsequent lower layers.

8. Small differences in pycnidium production were observed between soils collected from Mile 1019, Alaska Highway, Yukon Territory, and Lacombe, Alberta during May or August.

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